TITLE

NOVEL BRAIN-LOCALIZED PROTEIN KINASES HOMOLOGOUS TO HOMEODOMAIN-INTERACTING PROTEIN KINASES

[0001] This application claims the benefit of U.S. Provisional Application Ser. No. 60/456,958, filed March 25, 2003, and U.S. Provisional Application Ser. No. 60/491,251, filed July 31, 2003, both of which are incorporated herein by reference in their entireties.

BACKGROUND OF THE INVENTION

Field of the Invention

[0002] The present invention is directed to novel proteins homologous to homeodomain-interacting protein kinases (HIPKs), as well as nucleic acid molecules encoding such novel proteins.

[0003] Kinases are signal transmission proteins that regulate many different cell processes (e.g., proliferation, differentiation, and signaling) by adding phosphate groups to proteins. Uncontrolled signaling has been implicated in a variety of disease conditions including inflammation, cancer, arteriosclerosis, and psoriasis. Reversible protein phosphorylation is one of the main strategies for controlling activities of eukaryotic cells. It is estimated that more than 1000 of the 10,000

proteins active in a typical mammalian cell are phosphorylated. The high-energy phosphate, which drives activation, is generally transferred from adenosine triphosphate (ATP) molecules to a particular protein by protein kinases and removed from that protein by protein phosphatases. Phosphorylation occurs in response to extracellular signals (e.g., hormones, neurotransmitters, growth and differentiation factors), cell cycle checkpoints, and environmental or nutritional stresses, and is roughly analogous to turning on a molecular switch. When the switch goes on, the appropriate protein kinase activates, e.g., a metabolic enzyme, regulatory protein, receptor, cytoskeletal protein, ion channel or pump, or transcription factor.

[0004] Malfunctions of cellular signaling have been associated with many diseases. Regulation of signal transduction by cytokines and association of signal molecules with protooncogenes and tumor suppressor genes have been the subjects of intense research. Many therapeutic strategies can now be developed through the synthesis of compounds that activate or inactivate protein kinases (Sridhar et al. (2000) *Pharm. Res.* 17:1345-53).

[0005] The importance of kinases in the etiology of diseases has been well established. Kinase proteins are a major target for drug action and development. In January 2002 there were more than 100 clinical trials involving the modulation of kinases ongoing in the USA (Dumas (2001) *Curr. Opin. Drug Discov. Devel.* 4:378-89; Levitzki and Gazit (1995) *Science* 267:1782-88). Trials are ongoing in a wide variety of therapeutic indications including asthma, Parkinson's disease, inflammation, psoriasis, rheumatoid arthritis, spinal cord injuries, muscle conditions, osteoporosis, graft-versus-host disease, cardiovascular disorders, autoimmune disorders, retinal detachment, stroke, epilepsy, ischemia/reperfusion, breast cancer, ovarian cancer, glioblastoma, non-Hodgkin's lymphoma, colorectal cancer, non-small cell lung cancer, brain cancer, Kaposi's sarcoma, pancreatic cancer, various solid tumors, liver cancer, and other tumors. Numerous kinds of modulators of kinase activity are currently in clinical trials, including antisense molecules, antibodies, small molecules, and gene therapy.

[0006] Accordingly, it is valuable to the field of pharmaceutical development to identify and characterize previously unknown members of the kinase family. The present invention advances the state of the art by providing previously unidentified human kinase proteins which are structurally related to homeodomain-interacting protein kinases (HIPKs). HIPKs (HIPK1, HIPK2, and HIPK3) were originally identified via a yeast two-hybrid screen and shown to be nuclear serine/threonine kinases that function as transcriptional corepressors for homeodomain transcription factors (Kim et al. (1998) *J. Biol. Chem.* 273:25875-79). Although HIPK1 was originally identified as a homeodomain-interacting protein, the primary activities of HIPK2 and HIPK3 appear to be in pathways of cell death or proliferation. For example, HIPK2 was recently shown to regulate the proapoptotic function of p53 (Hofmann et al. (2002) *Nature Cell Biol.* 4:1-10; D'Orazi et al. (2002) *Nature Cell Biol.* 4:11-19), while HIPK3 was shown to bind Fas and induce FADD phosphorylation, thereby promoting formation of a HIPK3/FADD/Fas complex (Rochat-Steiner et al. (2000) *J. Exp. Med.* 192:1165-74).

[0007] Many therapeutic strategies are aimed at critical components in signal transduction pathways. Approaches for regulating kinase gene expression include specific antisense oligonucleotides for inhibiting posttranscriptional processing of messenger RNA, naturally occurring products and their chemical derivatives to inhibit kinase activity, and monoclonal antibodies to inhibit receptor-linked kinases. In some cases, kinase inhibitors also allow other therapeutic agents additional time to become effective and act synergistically with current treatments (Sridhar et al., *supra*).

[0008] Among the areas of pharmaceutical research that are currently receiving a great deal of attention are the role of phosphorylation in transcriptional control, apoptosis, protein degradation, nuclear import and export, cytoskeletal regulation, and checkpoint signaling (Hunter (1998-99) *Harvey Lect.* 94:81-119). The accumulating knowledge about signaling networks and the proteins involved will be put to practical use in the development of potent and specific pharmacological modulators of phosphorylation-dependent signaling that can be used for therapeutic purposes. The rational structure-based design and development of

highly specific kinase modulators is becoming routine, and drugs that intercede in signaling pathways are becoming a major class of drugs.

[0009] The kinases comprise one of the largest known protein groups, a superfamily of enzymes with widely varied functions and specificities. They are usually named after their substrate, their regulatory molecules, or some aspect of a mutant phenotype. With regard to substrates, the protein kinases may be roughly divided into two groups: those that phosphorylate serine or threonine residues (serine/threonine kinases; STKs), and those that phosphorylate tyrosine residues (protein tyrosine kinases; PTKs). A few protein kinases have dual specificity and phosphorylate threonine and tyrosine residues. Almost all kinases contain a similar 250-300 amino acid catalytic domain. The N-terminal domain, which contains subdomains I - IV, generally folds into a two-lobed structure, which binds and orients the ATP (or GTP) donor molecule. The larger C-terminal lobe, which contains subdomains VI A - XI, binds the protein substrate and carries out the transfer of the gamma phosphate from ATP to the hydroxyl group of a serine, threonine, or tyrosine residue. Subdomain V spans the two lobes.

[0010] The two groups of kinases may be further categorized into families by the different amino acid sequences (generally between 5 and 100 residues) located on either side of, or inserted into loops of, the kinase domain. These added amino acid sequences allow for the regulation of each kinase as it recognizes and interacts with its target protein. The primary structure of the kinase domains is conserved and can be further subdivided into eleven (I - XI) subdomains. Each of these eleven subdomains contains specific residues and motifs or patterns of amino acids that are characteristic of that subdomain and are highly conserved (Hardie and Hanks (1995) *The Protein Kinase Facts Book*, Vol. I, pp. 7-20, Academic Press, San Diego, CA).

Serine/Threonine Kinases

[0011] The second messenger-dependent protein kinases primarily mediate the effects of second messengers such as cyclic AMP (cAMP), cyclic GMP, inositol

triphosphate, phosphatidylinositol, 3,4,5-triphosphate, cyclic-ADP ribose, arachidonic acid, diacylglycerol and calcium-calmodulin. The cAMP-dependent protein kinases (PKA) are important members of the STK family. Cyclic AMP is an intracellular mediator of hormone action in all prokaryotic and animal cells that have been studied. Such hormone-induced cellular responses include thyroid hormone secretion, cortisol secretion, progesterone secretion, glycogen breakdown, bone resorption, and regulation of heart rate and force of heart muscle contraction. PKA is found in all animal cells and is thought to account for the effects of cAMP in most of these cells. Altered PKA expression is implicated in a variety of disorders and diseases including cancer, thyroid disorders, diabetes, atherosclerosis, and cardiovascular disease (Isselbacher, K.J. et al. (1994) *Harrison's Principles of Internal Medicine*, e.g., pp. 416-31, McGraw-Hill, New York, NY).

[0012] Calcium-calmodulin (CaM)-dependent protein kinases (CaM kinases) are also members of the STK family. Calmodulin is a calcium receptor that mediates many calcium-regulated processes by binding to target proteins in response to the binding of calcium. The principle target protein in these processes is CaM kinase. CaM kinases are involved in regulation of smooth muscle contraction (MLC kinase), glycogen breakdown (phosphorylase kinase), and neurotransmission (CaM kinase I and CaM kinase II). CaM kinase I phosphorylates a variety of substrates including the neurotransmitter-related proteins synapsin I and II, the gene transcription regulator, CREB, and the cystic fibrosis conductance regulator protein, CFTR (Haribabu et al. (1995) *EMBO J.* 14:3679-86). CaM kinase II also phosphorylates synapsin at different sites, and controls the synthesis of catecholamines in the brain through phosphorylation and activation of tyrosine hydroxylase. Many of the CaM kinases are activated by phosphorylation in addition to binding to CaM. The kinase may autophosphorylate itself, or may be phosphorylated by another kinase as part of a kinase cascade.

[0013] Another ligand-activated protein kinase is 5'-AMP-activated protein kinase (AMPK) (Gao et al. (1996) *J. Biol. Chem.* 271:8675-81). Mammalian AMPK is a regulator of fatty acid and sterol synthesis through phosphorylation of the enzymes

acetyl-CoA carboxylase and hydroxymethylglutaryl (HMG)-CoA reductase, and mediates responses of these pathways to cellular stresses such as heat shock and depletion of glucose and ATP. AMPK is a heterotrimeric complex comprised of a catalytic alpha subunit and two noncatalytic beta and gamma subunits that are believed to regulate the activity of the alpha subunit. Subunits of AMPK have a much wider distribution in nonlipogenic tissues (such as brain, heart, spleen, and lung) than expected. This distribution suggests that its role may extend beyond regulation of lipid metabolism alone.

[0014] The mitogen-activated protein kinases (MAP kinases) are also members of the STK family. MAP kinases also regulate intracellular signaling pathways. They mediate signal transduction from the cell surface to the nucleus via phosphorylation cascades. Several subgroups have been identified, and each manifests different substrate specificities and responds to distinct extracellular stimuli (Egan and Weinberg (1993) *Nature* 365:781-83). MAP kinase signaling pathways are present in mammalian cells as well as in yeast. The extracellular stimuli that activate mammalian pathways regulated by MAP kinases include epidermal growth factor (EGF), ultraviolet light, hyperosmolar medium, heat shock, endotoxic lipopolysaccharide (LPS), and proinflammatory cytokines such as tumor necrosis factor (TNF) and interleukin-1 (IL-1).

[0015] EGF receptor (EGFR) is found in over half of breast tumors unresponsive to hormone therapy. EGF is found in many tumors, and EGF may be required for tumor cell growth. Antibody to EGF was shown to block the growth of tumor xenografts in mice, while an antisense oligonucleotide for amphiregulin inhibited growth of a pancreatic cancer cell line.

[0016] Tamoxifen, a protein kinase C inhibitor with antiestrogen activity, is currently a standard treatment for hormone-dependent breast cancer. The use of this compound may increase the risk of developing cancer in other tissues such as the endometrium. Raloxifene, a related compound, has been shown to protect against osteoporosis. The tissue specificity of inhibitors must be considered when identifying therapeutic targets.

[0017] Cell proliferation and differentiation in normal cells are under the regulation and control of multiple MAP kinase cascades. Aberrant and deregulated functioning of MAP kinases can initiate and support carcinogenesis. Insulin and insulin-like growth factor-I (IGF-I) also activate a mitogenic MAP kinase pathway that may be important in acquired insulin resistance occurring in type 2 diabetes.

[0018] Many cancers become refractory to chemotherapy by developing a survival strategy involving the constitutive activation of the phosphatidylinositol-3 (PI-3) kinase-protein kinase B/Akt signaling cascade. This survival-signaling pathway thus becomes an important target for the development of specific inhibitors that would block its function. PI-3 kinase/Akt signaling is equally important in diabetes. The pathway activated by receptor tyrosine kinases (RTKs) subsequently regulates glycogen synthase 3, producing alterations in glycogen synthesis and glucose uptake. Since Akt has decreased activity in type 2 diabetes, it provides a therapeutic target.

[0019] Protein kinase inhibitors provide much of our knowledge about regulation and coordination of physiological functions. Endogenous peptide inhibitors occur *in vivo*. A pseudosubstrate sequence within PKC acts to inhibit the kinase in the absence of its lipid activator. A PKC inhibitor such as chelerythrine acts on the catalytic domain to block substrate interaction, while calphostin acts on the regulatory domain to mimic the pseudosubstrate sequence and block ATPase activity, or by inhibiting cofactor binding. The ability to inhibit specific PKC isozymes is limited.

[0020] Although some protein kinases have, to date, no known system of physiological regulation, many are activated or inactivated by autophosphorylation or phosphorylation by upstream protein kinases. The regulation of protein kinases also occurs transcriptionally, posttranscriptionally, and posttranslationally. The mechanism of posttranscriptional regulation is alternative splicing of precursor mRNA. Protein kinase C-β1 and -βII are two isoforms of a single PKC-β gene derived from differences in the splicing of the exon encoding the C-terminal 50-52 amino acids. Splicing can be regulated by a kinase cascade in response to peptide

hormones such as insulin and IGF-I. PKC-βI and -βII have different specificities for phosphorylating members of the MAP kinase family, for glycogen synthase 3b, for nuclear transcription factors such as TLS/Fus, and for other nuclear kinases. By inhibiting the posttranscriptional alternative splicing of PKC-βII mRNA, PKC-βII-dependent processes are inhibited.

[0021] The development of antisense oligonucleotides to inhibit the expression of various protein kinases has been successful. Antisense oligonucleotides are short lengths of synthetically manufactured, chemically modified DNA or RNA designed to specifically interact with mRNA transcripts encoding target proteins. The interaction of the antisense moiety with mRNA inhibits protein translation and, in some cases, posttranscriptional processing (e.g., alternative splicing and stability) of mRNA. Antisense oligonucleotides have been developed to alter alternative splicing of bcl-xlong to short mRNA forms and for inhibiting the translation of PKC-α and PKC-ζ.

[0022] Protein kinase C isoforms have been implicated in cellular changes observed in the vascular complications of diabetes. Hyperglycemia is associated with increased levels of PKC-α and -β isoforms in renal glomeruli of diabetic rats. Oral administration of a PKC-β inhibitor prevented the increased mRNA expression of TGF-β1 and extracellular matrix component genes. Administration of the specific PKC-β inhibitor (LY333531) also normalized levels of cytokines, caldesmon and hemodynamics of retinal and renal blood flow. Overexpression of the PKC-β isoform in the myocardium resulted in cardiac hypertrophy and failure. The use of LY333531 to prevent adverse effects of cardiac PKC-β overexpression in diabetic subjects is under investigation. The compound is also in Phase II/III clinical trials for diabetic retinopathy and diabetic macular edema indicating that it may be pharmacodynamically active.

[0023] PRK (proliferation-related kinase) is a serum/cytokine inducible STK that is involved in regulation of the cell cycle and cell proliferation in human megakaryocytic cells (Li et al. (1996) *J. Biol. Chem.* 271:19402-08). PRK is related to the polo family of STKs implicated in cell division. PRK is

downregulated in lung tumor tissue and may be a protooncogene whose deregulated expression in normal tissue leads to oncogenic transformation. Altered PRK expression is implicated in a variety of disease conditions including cancer, inflammation, immune disorders, and disorders affecting growth and development.

[0024] DNA-dependent protein kinase (DNA-PK) is involved in the repair of double-strand breaks in mammalian cells. This enzyme requires ends of double-stranded DNA or transitions from single-stranded to double-stranded DNA in order to act as a serine/threonine kinase. Cells with defective or deficient DNA-PK activity are unable to repair radiation-induced DNA double-strand breaks and consequently are very sensitive to the lethal effects of ionizing radiation. Inhibition of DNA-PK has the potential to increase the efficacy of antitumor treatment with radiation or chemotherapeutic agents.

[0025] The cyclin-dependent protein kinases (CDKs) are another group of STKs that control the progression of cells through the cell cycle. Cyclins are small regulatory proteins that act by binding to and activating CDKs that then trigger various phases of the cell cycle by phosphorylating and activating selected proteins involved in the mitotic process. CDKs are unique in that they require multiple inputs to become activated. In addition to the binding of cyclin, CDK activation requires the phosphorylation of a specific threonine residue and the dephosphorylation of a specific tyrosine residue.

[0026] Cellular inhibitors of CDKs play a major role in cell-cycle progression. Alterations in the expression, function, and structure of cyclin and CDK are encountered in the cancer phenotype. Therefore CDKs may be important targets for new cancer therapeutic agents.

[0027] Often chemotherapy resistant cells tend to escape apoptosis. Under certain circumstances, inappropriate CDK activation may even promote apoptosis by encouraging the progression of the cell cycle under unfavorable conditions, e.g., attempting mitosis while DNA damage is largely unrepaired.

[0028] Purines and purine analogs act as CDK inhibitors. Flavopiridol (L86-8275) is a flavonoid that causes 50% growth inhibition of tumor cells at 25-160nM (Kaur et al. (1992) *J. Natl. Cancer Inst.* 84:1736-40). It also inhibits EGFR and PKA (IC_{50}: approximately 100μM). Flavopiridol induces apoptosis and inhibits lymphoid, myeloid, colon, and prostate cancer cells grown *in vivo* as tumor xenografts in nude mice.

[0029] Staurosporine and its derivative, UCN-01, in addition to inhibiting protein kinase C, inhibit cyclin B/CDK (IC_{50}: 3-6nM). Staurosporine is toxic, but its derivative 7-hydroxystaurosporine (UCN-01) has antitumor properties and is in clinical trials. UCN-01 affects the phosphorylation of CDKs and alters the cell-cycle checkpoint functioning. These compounds illustrate that multiple intracellular targets may be affected as the concentration of an inhibitor is increased within cells.

Protein Tyrosine Kinases

[0030] Protein tyrosine kinases (PTKs) specifically phosphorylate tyrosine residues on their target proteins and may be divided into transmembrane, receptor PTKs (RTKs) and nontransmembrane, nonreceptor PTKs. Transmembrane protein tyrosine kinases are receptors for most growth factors. Binding of growth factor to the receptor activates the transfer of a phosphate group from ATP to selected tyrosine side chains of the receptor and other specific proteins. Growth factors (GFs) associated with receptor PTKs include epidermal GF (EGF), platelet-derived GF (PDGF), fibroblast GF (FGF), hepatocyte GF (HGF), insulin and insulin-like GFs (IGFs), nerve GF (NGF), vascular endothelial GF (VEGF), and macrophage colony stimulating factor (MCSF).

[0031] Because RTKs stimulate tumor cell proliferation, inhibitors of RTKs may inhibit the growth and proliferation of cancers by preventing tumor angiogenesis and can eliminate support from the host tissue by targeting RTKs located on vascular cells (e.g., blood vessel endothelial cells (VEGF receptor, or VEGFR) and stromal fibroblasts (FGF receptor)).

[0032] VEGF stimulates endothelial cell growth during angiogenesis, and increases the permeability of tumor vasculature so that proteins and other growth factors become accessible to the tumor. Broad-spectrum antitumor efficacy of an oral dosage form of an inhibitor of VEGF signaling has been reported. Thus, inhibition of VEGF receptor signaling presents an important therapeutic target. An extracellular receptor can also be a target for inhibition. For example, the EGF receptor family and its ligands are overexpressed and exist as an autocrine loop in many tumor types.

[0033] Increasing knowledge of the structure and activation mechanism of RTKs and the signaling pathways controlled by tyrosine kinases provided the possibility for the development of target-specific drugs and new anticancer therapies. Approaches towards the prevention or interception of deregulated RTK signaling include the development of selective components that target either the extracellular ligand-binding domain or the intracellular tyrosine kinase or substrate-binding region.

[0034] One of the most successful strategies for selectively killing tumor cells is the use of monoclonal antibodies (mAbs) that are directed against the extracellular domain of RTKs that are critically involved in cancer and are expressed at the surface of tumor cells. In recent years, recombinant antibody technology has made enormous progress in the design, selection and production of newly engineered antibodies, and it is possible to generate humanized antibodies, human-mouse chimeric or bispecific antibodies for targeted cancer therapy. Mechanistically, anti-RTK mAbs might work by blocking the ligand-receptor interaction and therefore inhibiting ligand-induced RTK signaling and increasing RTK downregulation and internalization. In addition, the binding of mAbs to certain epitopes on the cancer cells induces immune-mediated responses, such as opsonization and complement-mediated lysis, and triggers antibody-dependent cellular cytotoxicity (ADCC) by macrophages or natural killer cells. In recent years, it has become evident that mAbs control tumor growth by altering the intracellular signaling pattern inside the targeted tumor cell, leading to growth inhibition and/or apoptosis. In contrast, bispecific antibodies can bridge selected

surface molecules on a target cell with receptors on an effector cell triggering cytotoxic responses against the target cell. Despite the toxicity that has been seen in clinical trials of bispecific antibodies, advances in antibody engineering, characterization of tumor antigens, and immunology might help to predict rationally designed bispecific antibodies for anticancer therapy.

[0035] Another promising approach to inhibit aberrant RTK signaling are small molecule drugs that selectively interfere with the intrinsic tyrosine kinase activity and thereby block receptor autophosphorylation and activation of downstream signal transducers. The tyrphostins, which belong to the quinazolines, are one important group of such inhibitors that compete with ATP for the ATP binding site at the tyrosine kinase domain of the receptor; some members of the tyrphostins have been shown to specifically inhibit the EGFR. Potent and selective inhibitors of receptors involved in neovascularization have also been developed and are now undergoing clinical evaluation. Using the advantages of structure-based drug design, crystallographic structure information, combinatorial chemistry, and high-throughput screening, new structural classes of tyrosine kinase inhibitors (TKIs) with increased potency and selectivity, higher *in vitro* and *in vivo* efficacy, and decreased toxicity have emerged.

[0036] Recombinant immunotoxins provide another possibility of target-selective drug design. They are composed of a bacterial or plant toxin either fused or chemically conjugated to a specific ligand, such as the variable domains of the heavy and light chains of mAbs, or to a growth factor. For example, immunotoxins may contain the bacterial toxins *Pseudomonas* exotoxin A or diphtheria toxin, or the plant toxins ricin A or clavin. These recombinant molecules can selectively kill their target cells when internalized after binding to specific cell-surface receptors.

[0037] The use of antisense oligonucleotides represents another strategy to inhibit the activation of RTKs. Antisense oligonucleotides are short pieces of synthetic DNA or RNA that are designed to interact with the mRNA to block the transcription and thus the expression of specific proteins. These compounds

interact with the mRNA by Watson-Crick base pairing and are therefore highly specific for the target protein. On the other hand, bioavailability may be poor since oligonucleotides can be degraded upon internalization by cellular endonucleases and exonucleases. Nevertheless, several preclinical and clinical studies suggest that antisense therapy might be therapeutically useful for the treatment of solid tumors.

[0038] A recent search of a public website cataloging clinical trials, many of which are actively recruiting patients, provided a list of over 100 clinical trials in response to the search term "kinase." A summary of some of the most successful drugs against receptor tyrosine kinase (RTK) signaling that are currently being evaluated in clinical phases or have already been approved by the FDA is shown in Table 1.

TABLE 1

RTK	Drug	Company	Description	Status
human epidermal growth factor receptor-2 (HER2)	trastuzumab (Herceptin®)	Genentech	mAb directed against HER2	Approved in 1998
EGFR	gefitinib (Iressa®; ZD1839)	AstraZeneca	TKI that inhibits EGFR signaling	Approved in 2003
EGFR	cetuximab (Erbitux®; C225)	ImClone	mAb directed against EGFR	Approved in 2004
EGFR	erlotinib (Tarceva®; OSI-774)	OSI Pharmaceuticals	TKI that inhibits EGFR signaling	Phase II/III
VEGFR2	investigational oral drug (name not available)	Pfizer	TKI that inhibits VEGFR2 signaling	Phase I/II
VEGFR2	ZD6474	AstraZeneca	TKI that inhibits VEGFR2 signaling	Phase II
PDGFR	imatinib mesylate (Gleevec®; STI571)	Novartis	TKI that inhibits PDGFR signaling (among several other actions)	Approved in 2001/ 2002

[0039] Therefore, the potential of RTKs and their relevant signaling as selective anticancer targets for therapeutic intervention has been recognized. As a consequence, a variety of successful target-specific drugs such as mAbs and RTK inhibitors have been developed and are currently being evaluated in clinical trials.

[0040] Nonreceptor PTKs lack transmembrane regions and instead form complexes with the intracellular regions of cell surface receptors. Such receptors that function through nonreceptor PTKs include those for cytokines, hormones (e.g., growth hormone and prolactin) and antigen-specific receptors on T and B lymphocytes.

[0041] Many of these PTKs were first identified as the products of mutant oncogenes in cancer cells where their activation was no longer subject to normal cellular controls. About one third of the known oncogenes encode PTKs, and it is well known that cellular transformation (oncogenesis) is often accompanied by increased tyrosine phosphorylation activity (Charbonneau and Tonks (1992) *Annu. Rev. Cell. Biol.*, 8:463-93).

[0042] Many tyrosine kinase inhibitors are derived from natural products including flavopiridol, genistein, erbstatin, lavendustin A, staurosporine, and UCN-01. Inhibitors directed at the ATP binding site are also available. Signals from RTKs can also be inhibited at other target sites, such as nuclear tyrosine kinases, membrane anchors (inhibition of farnesylation), and transcription factors.

[0043] Targeting the signaling potential of growth-promoting tyrosine kinases such as EGFR, HER2, PDGFR, *src*, and *abl* will block tumor growth, whereas blocking IGF-I and TRK will interfere with tumor cell survival. Inhibiting these kinases will lead to tumor shrinkage and apoptosis. Fkl-1/KDR and *src* are kinases necessary for neovascularization (angiogenesis) of tumors, and inhibition of these kinases will slow tumor growth, thereby decreasing metastases.

[0044] Inhibitors of RTKs stabilize the tumor in terms of cell proliferation, allowing normal cell loss via apoptosis, and prevent cell migration, invasion, and metastases. These drugs are likely to increase the time required for tumor progression, and may inhibit or attenuate the aggressiveness of the disease, but may not initially result in measurable tumor regression.

[0045] An example of cancer arising from a defective tyrosine kinase is a class of anaplastic lymphoma kinase (ALK)-positive lymphomas referred to as

"ALKomas," which display inappropriate expression of a neural-specific tyrosine kinase.

[0046] Iressa® (gefitinib; ZD1839) is an orally active, selective EGFR inhibitor. This compound disrupts signaling involved in cancer cell proliferation, cell survival and tumor growth support by the host. Clinical trials with this agent demonstrated that it was clinically efficacious and well tolerated by patients. The compound has shown promising cytotoxicity towards several cancer cell lines. It recently has been approved by the FDA for advanced nonsmall-cell lung cancer.

[0047] Many growth factors and cytokines regulate cellular functions via the Janus kinase (JAK) signal transducers and activators of transcription (Stat). The JAK inhibitor tyrphostin AG490 prevents Stat3 activation and suppresses the growth of human prostate cancer cells.

[0048] Since the majority of protein kinases, both STKs and PTKs, are expressed in the brain, often in neuron-specific fashion, protein phosphorylation must play a key role in the development, maintenance, and function of the vertebrate central nervous system (Hunter (1998-99) *Harvey Lect.* 94:81-119). There is substantial evidence that many neurological disorders, such as Alzheimer's disease, Parkinson's disease, Huntington's disease, age-related neurodegeneration, depression, bipolar disorder, and obsessive-compulsive disorder, result from deficiencies in neurogenesis itself, or in the balance between neurogenesis and neurodegeneration (Duman et al. (1999) *Biol. Psychiatry* 46:1181-91; Jacobs et al. (2000) *Mol. Psychiatry* 5:262-69; Gage (2002) *J. Neurosci.* 22:612-13). Thus, neuron-specific kinases are well established as targets for the development of pharmacologically active modulators useful in the treatment of neurological diseases.

SUMMARY OF THE INVENTION

[0049] The invention provides isolated protein kinase polypeptides and the isolated nucleic acid molecules that encode them. The invention also provides genetically

engineered expression vectors, host cells, and transgenic animals comprising the nucleic acid molecules of the invention. The invention additionally provides antisense and RNA interference (RNAi) molecules to the nucleic acid molecules of the invention. The invention further provides inhibitors, activators, and antibodies capable of binding to the protein kinase polypeptides of the invention, and provides uses for these inhibitors, activators, and antibodies in the prevention and treatment of neurological disorders.

BRIEF DESCRIPTION OF THE DRAWINGS

[0050] FIG. **1** shows preferred siRNA molecules targeted to human HIPK4 mRNA for use in RNAi. Target segments [SEQ ID NOs:18-34; 69-88; 129-143; and 174-179] of the HIPK4 transcripts are grouped according to their first two nucleotides (AA, CA, GA, or TA) and are shown in the 5'->3' orientation. "GC Ratio" refers to the percentage of total G+C nucleotides in each target segment; "Position" refers to the nucleotide position in the human HIPK4 cDNA (SEQ ID NO:1) immediately preceding the beginning of each target segment. Preferred siRNA molecules (siRNA duplexes) are shown on the right side of the figure. The sense strand for each siRNA duplex [SEQ ID NOs:35-51; 89-108; 144-158; and 180-185] is shown in the 5'->3' orientation; the corresponding antisense strand [SEQ ID NOs:52-68; 109-128; 159-173; and 186-191] is shown in the 3'->5' orientation. For example, the siRNA molecule directed to the first target segment presented in the figure (i.e., SEQ ID NO:18) is the siRNA duplex of the sense and antisense strands identified (i.e., SEQ ID NO:35 and SEQ ID NO:52, respectively).

[0051] FIG. **2A**, **2B**, **2C**, and **2D** show the predicted amino acid sequences of human HIPK4 (SEQ ID NO:2), its predicted ATP binding domain, its predicted serine/threonine binding domain, and its predicted protein kinase domain, respectively.

[0052] FIG. **3A**, **3B**, **3C**, and **3D** show the predicted amino acid sequences of mouse HIPK4 (SEQ ID NO:5), its predicted ATP binding domain, its predicted

serine/threonine binding domain, and its predicted protein kinase domain, respectively.

[0053] FIG. **4A**, **4B**, **4C**, and **4D** show the predicted amino acid sequences of monkey HIPK4 (SEQ ID NO:8), its predicted ATP binding domain, its predicted serine/threonine binding domain, and its predicted protein kinase domain, respectively.

[0054] FIG. **5A**, **5B**, **5C**, and **5D** show the predicted amino acid sequences of rat HIPK4 (SEQ ID NO:17), its predicted ATP binding domain, its predicted serine/threonine binding domain, and its predicted protein kinase domain, respectively.

[0055] FIG. **6** shows a comparison of the amino acid sequences (FIG. **6A**) of the conserved kinase catalytic domains of human HIPK4 (humHIPK4; from SEQ ID NO:2), mouse HIPK4 (musHIPK4; from SEQ ID NO:5), monkey HIPK4 (monkeyHIPK4; from SEQ ID NO:8), and rat HIPK4 (ratHIPK4; from SEQ ID NO:17) with the kinase catalytic domains of several known HIPKs (musHIPK3; ratHIPK3; humHIPK3; humHIPK1-like; musHIPK1; humHIPK2; musHIPK2; wormF20B6; yeastYAK1; and dmCG17090). A phylogenetic tree of the aligned sequences, including public accession codes/numbers, is presented in FIG. **6B**.

[0056] FIG. **7** shows a comparison of the amino acid sequences (FIG. **7A**) of the conserved kinase catalytic domains of human HIPK4 (humHIPK4; from SEQ ID NO:2), mouse HIPK4 (musHIPK4; from SEQ ID NO:5), monkey HIPK4 (monkeyHIPK4; from SEQ ID NO:8), and rat HIPK4 (ratHIPK4; from SEQ ID NO:17) with the kinase catalytic domains of several known DYRKs, or dual-specificity tyrosine phosphorylated and regulated kinases (musDYRK1A; ratDYRK1A; humDYRK1A; humDYRK1B; musDYRK1B; dmMNB; wormT04C10; dmSMI35A; humDYRK4; humDYRK2; humDYRK3; wormF49E11; KAB7_SCHPO; and POM1_SCHPO). A phylogenetic tree of the aligned sequences, including public accession codes/numbers, is presented in FIG. **7B**.

DETAILED DESCRIPTION OF THE INVENTION

[0057] The present invention provides novel isolated and purified polynucleotides and polypeptides homologous to the kinase catalytic domains of various known HIPKs.

[0058] For example, the invention provides purified and isolated polynucleotides encoding a novel protein kinase, herein designated "HIPK4." This protein kinase has an approximate molecular weight of 69 kD. Preferred DNA sequences of the invention include genomic and cDNA sequences and chemically synthesized DNA sequences.

[0059] The nucleotide sequence of a cDNA encoding this novel protein kinase, designated human HIPK4 cDNA, is set forth in SEQ ID NO:1. Polynucleotides of the present invention also include polynucleotides that hybridize under stringent conditions to SEQ ID NO:1, or its complement, and/or encode polypeptides that retain substantial biological activity of full-length human HIPK4. Polynucleotides of the present invention also include continuous portions of the sequence set forth in SEQ ID NO:1 comprising at least 21 consecutive nucleotides.

[0060] The deduced amino acid sequence of human HIPK4 is set forth in SEQ ID NO:2. Polypeptides of the present invention also include continuous portions of the sequence set forth in SEQ ID NO:2 comprising at least seven consecutive amino acids. A preferred polypeptide of the present invention includes any continuous portion of the sequence set forth in SEQ ID NO:2 that retains substantial biological activity (i.e., an active fragment) of full-length human HIPK4. Polynucleotides of the present invention also include, in addition to those polynucleotides of human origin described above, polynucleotides that encode the amino acid sequence set forth in SEQ ID NO:2 or a continuous portion thereof, and that differ from the polynucleotides of human origin described above only due to the well-known degeneracy of the genetic code.

[0061] The nucleotide sequence of a genomic DNA encoding this novel protein kinase, designated human HIPK4 genomic DNA, is set forth in SEQ ID NO:3. Polynucleotides of the present invention also include polynucleotides that hybridize under stringent conditions to SEQ ID NO:3, or its complement, and/or encode polypeptides that retain substantial biological activity of full-length human HIPK4. Polynucleotides of the present invention also include continuous portions of the sequence set forth in SEQ ID NO:3 comprising at least 21 consecutive nucleotides.

[0062] The nucleotide sequence of a cDNA encoding this novel protein kinase, designated mouse HIPK4 cDNA, is set forth in SEQ ID NO:4. Polynucleotides of the present invention also include polynucleotides that hybridize under stringent conditions to SEQ ID NO:4, or its complement, and/or encode polypeptides that retain substantial biological activity of full-length mouse HIPK4. Polynucleotides of the present invention also include continuous portions of the sequence set forth in SEQ ID NO:4 comprising at least 21 consecutive nucleotides.

[0063] The deduced amino acid sequence of mouse HIPK4 is set forth in SEQ ID NO:5. Polypeptides of the present invention also include continuous portions of the sequence set forth in SEQ ID NO:5 comprising at least seven consecutive amino acids. A preferred polypeptide of the present invention includes any continuous portion of the sequence set forth in SEQ ID NO:5 that retains substantial biological activity (i.e., an active fragment) of full-length mouse HIPK4. Polynucleotides of the present invention also include, in addition to those polynucleotides of mouse origin described above, polynucleotides that encode the amino acid sequence set forth in SEQ ID NO:5 or a continuous portion thereof, and that differ from the polynucleotides of mouse origin described above only due to the well-known degeneracy of the genetic code.

[0064] The nucleotide sequence of a genomic DNA encoding this novel protein kinase, designated mouse HIPK4 genomic DNA, is set forth in SEQ ID NO:6. Polynucleotides of the present invention also include polynucleotides that hybridize under stringent conditions to SEQ ID NO:6, or its complement, and/or

encode polypeptides that retain substantial biological activity of full-length mouse HIPK4. Polynucleotides of the present invention also include continuous portions of the sequence set forth in SEQ ID NO:6 comprising at least 21 consecutive nucleotides.

[0065] The nucleotide sequence of a cDNA encoding this novel protein kinase, designated monkey HIPK4 cDNA, is set forth in SEQ ID NO:7. Polynucleotides of the present invention also include polynucleotides that hybridize under stringent conditions to SEQ ID NO:7, or its complement, and/or encode polypeptides that retain substantial biological activity of full-length monkey HIPK4. Polynucleotides of the present invention also include continuous portions of the sequence set forth in SEQ ID NO:7 comprising at least 21 consecutive nucleotides.

[0066] The deduced amino acid sequence of monkey HIPK4 is set forth in SEQ ID NO:8. Polypeptides of the present invention also include continuous portions of the sequence set forth in SEQ ID NO:8 comprising at least seven consecutive amino acids. A preferred polypeptide of the present invention includes any continuous portion of the sequence set forth in SEQ ID NO:8 that retains substantial biological activity (i.e., an active fragment) of full-length monkey HIPK4. Polynucleotides of the present invention also include, in addition to those polynucleotides of monkey origin described above, polynucleotides that encode the amino acid sequence set forth in SEQ ID NO:8 or a continuous portion thereof, and that differ from the polynucleotides of monkey origin described above only due to the well-known degeneracy of the genetic code.

[0067] The nucleotide sequence of a cDNA encoding this novel protein kinase, designated rat HIPK4 cDNA, is set forth in SEQ ID NO:16. Polynucleotides of the present invention also include polynucleotides that hybridize under stringent conditions to SEQ ID NO:16, or its complement, and/or encode polypeptides that retain substantial biological activity of full-length rat HIPK4. Polynucleotides of the present invention also include continuous portions of the sequence set forth in SEQ ID NO:16 comprising at least 21 consecutive nucleotides.

[0068] The deduced amino acid sequence of rat HIPK4 is set forth in SEQ ID NO:17. Polypeptides of the present invention also include continuous portions of the sequence set forth in SEQ ID NO:17 comprising at least seven consecutive amino acids. A preferred polypeptide of the present invention includes any continuous portion of the sequence set forth in SEQ ID NO:17 that retains substantial biological activity (i.e., an active fragment) of full-length rat HIPK4. Polynucleotides of the present invention also include, in addition to those polynucleotides of rat origin described above, polynucleotides that encode the amino acid sequence set forth in SEQ ID NO:17 or a continuous portion thereof, and that differ from the polynucleotides of rat origin described above only due to the well-known degeneracy of the genetic code.

[0069] The isolated polynucleotides of the present invention may be used as hybridization probes and primers to identify and isolate nucleic acids having sequences identical to, or similar to, those encoding the disclosed polynucleotides. Hybridization methods for identifying and isolated nucleic acids include polymerase chain reaction (PCR), Southern hybridization, and Northern hybridization, and are well known to those skilled in the art.

[0070] Hybridization reactions can be performed under conditions of different stringencies. The stringency of a hybridization reaction includes the difficulty with which any two nucleic acid molecules will hybridize to one another. Preferably, each hybridizing polynucleotide hybridizes to its corresponding polynucleotide under reduced stringency conditions, more preferably stringent conditions, and most preferably highly stringent conditions. Examples of stringency conditions are shown in Table 2 below: highly stringent conditions are those that are at least as stringent as, for example, conditions A-F; stringent conditions are at least as stringent as, for example, conditions G-L; and reduced stringency conditions are at least as stringent as, for example, conditions M-R.

TABLE 2

Stringency Condition	Poly-nucleotide Hybrid	Hybrid Length (bp)[1]	Hybridization Temperature and Buffer[2]	Wash Temperature and Buffer[2]
A	DNA:DNA	> 50	65°C; 1X SSC -or- 42°C; 1X SSC, 50% formamide	65°C; 0.3X SSC
B	DNA:DNA	<50	T_B*; 1X SSC	T_B*; 1X SSC
C	DNA:RNA	> 50	67°C; 1X SSC -or- 45°C; 1X SSC, 50% formamide	67°C; 0.3X SSC
D	DNA:RNA	<50	T_D*; 1X SSC	T_D*; 1X SSC
E	RNA:RNA	>50	70°C; 1X SSC -or- 50°C; 1X SSC, 50% formamide	70°C; 0.3xSSC
F	RNA:RNA	<50	T_F*; 1X SSC	T_f*; 1X SSC
G	DNA:DNA	>50	65°C; 4X SSC -or- 42°C; 4X SSC, 50% formamide	65°C; 1X SSC
H	DNA:DNA	<50	T_H*; 4X SSC	T_H*; 4X SSC
I	DNA:RNA	>50	67°C; 4X SSC -or- 45°C; 4X SSC, 50% formamide	67°C; 1X SSC
J	DNA:RNA	<50	T_J*; 4X SSC	T_J*; 4X SSC
K	RNA:RNA	>50	70°C; 4X SSC -or- 50°C; 4X SSC, 50% formamide	67°C; 1X SSC
L	RNA:RNA	<50	T_L*; 2X SSC	T_L*; 2X SSC
M	DNA:DNA	>50	50°C; 4X SSC -or- 40°C; 6X SSC, 50% formamide	50°C; 2X SSC
N	DNA:DNA	<50	T_N*; 6X SSC	T_N*; 6X SSC
O	DNA:RNA	>50	55°C; 4X SSC -or- 42°C; 6X SSC, 50% formamide	55°C; 2X SSC
P	DNA:RNA	<50	T_P*; 6X SSC	T_P*; 6X SSC

Stringency Condition	Poly-nucleotide Hybrid	Hybrid Length (bp)[1]	Hybridization Temperature and Buffer[2]	Wash Temperature and Buffer[2]
Q	RNA:RNA	>50	60°C; 4X SSC -or- 45°C; 6X SSC, 50% formamide	60°C; 2X SSC
R	RNA:RNA	<50	T_R*; 4X SSC	T_R*; 4X SSC

[1] The hybrid length is that anticipated for the hybridized region(s) of the hybridizing polynucleotides. When hybridizing a polynucleotide to a target polynucleotide of unknown sequence, the hybrid length is assumed to be that of the hybridizing polynucleotide. When polynucleotides of known sequence are hybridized, the hybrid length can be determined by aligning the sequences of the polynucleotides and identifying the region or regions of optimal sequence complementarity.

[2] SSPE (1xSSPE is 0.15M NaCl, 10mM NaH_2PO_4, and 1.25mM EDTA, pH 7.4) can be substituted for SSC (1xSSC is 0.15M NaCl and 15mM sodium citrate) in the hybridization and wash buffers; washes are performed for 15 minutes after hybridization is complete.

T_B* - T_R*: The hybridization temperature for hybrids anticipated to be less than 50 base pairs in length should be 5-10°C less than the melting temperature (T_m) of the hybrid, where T_m is determined according to the following equations. For hybrids less than 18 base pairs in length, T_m(°C) = 2(# of A + T bases) + 4(# of G + C bases). For hybrids between 18 and 49 base pairs in length, T_m(°C) = 81.5 + 16.6($\log_{10}Na^+$) + 0.41(%G + C) - (600/N), where N is the number of bases in the hybrid, and Na^+ is the concentration of sodium ions in the hybridization buffer (Na^+ for 1xSSC = 0.165M).

Additional examples of stringency conditions for polynucleotide hybridization are provided in Sambrook et al. (1989) *Molecular Cloning: A Laboratory Manual*, Chs. 9 & 11, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, and Ausubel et al., eds. (1995) *Current Protocols in Molecular Biology*, Sects. 2.10 & 6.3-6.4, John Wiley & Sons, Inc., herein incorporated by reference.

[0071] The isolated polynucleotides of the present invention may be used as hybridization probes and primers to identify and isolate DNAs having sequences encoding allelic variants of the disclosed polynucleotides. Allelic variants are naturally occurring alternative forms of the disclosed polynucleotides that encode polypeptides that are identical to or have significant similarity to the polypeptides encoded by the disclosed polynucleotides. Preferably, allelic variants have at least 90% sequence identity (more preferably, at least 95% identity; most preferably, at least 99% identity) with the disclosed polynucleotides. In one embodiment of the invention, an isolated nucleic acid molecule comprising a sequence at least 96.3% identical to SEQ ID NO:1 is provided.

[0072] The isolated polynucleotides of the present invention may also be used as hybridization probes and primers to identify and isolate DNAs having sequences encoding polypeptides homologous to the disclosed polynucleotides. These homologs are polynucleotides and polypeptides isolated from different species than those of the disclosed polypeptides and polynucleotides, or within the same species, but with significant sequence similarity to the disclosed polynucleotides and polypeptides. Preferably, polynucleotide homologs have at least 60% sequence identity (more preferably, at least 75% identity; most preferably, at least 90% identity) with the disclosed polynucleotides, whereas polypeptide homologs have at least 30% sequence identity (more preferably, at least 45% identity; most preferably, at least 60% identity) with the disclosed polypeptides. Preferably, homologs of the disclosed polynucleotides and polypeptides are those isolated from mammalian species. In one embodiment of the invention, an isolated nucleic acid molecule comprising a sequence at least 96.3% identical to SEQ ID NO:1 is provided. In another embodiment of the invention, a purified polypeptide, the amino acid sequence of which comprises a sequence at least 97.2% identical to SEQ ID NO:2, is provided.

[0073] The isolated polynucleotides of the present invention may also be used as hybridization probes and primers to identify cells and tissues that express the polypeptides of the present invention and the conditions under which they are expressed.

[0074] Additionally, the isolated polynucleotides of the present invention may be used to alter (i.e., enhance, reduce, or modify) the expression of the genes corresponding to the polynucleotides of the present invention in an organism. These "corresponding genes" are the genomic DNA sequences of the present invention (e.g., SEQ ID NO:3 or SEQ ID NO:6) that are transcribed to produce the mRNAs from which the cDNA polynucleotides of the present invention (e.g., SEQ ID NO:1 or SEQ ID NO:4) are derived.

[0075] Altered expression of the genes of the present invention in a cell or organism may be achieved through the use of various inhibitory polynucleotides,

such as antisense polynucleotides and ribozymes that bind and/or cleave the mRNA transcribed from the genes of the invention (e.g., Galderisi et al. (1999) *J. Cell Physiol.* 181:251-57; Sioud (2001) *Curr. Mol. Med.* 1:575-88).

[0076] The antisense polynucleotides or ribozymes of the invention can be complementary to an entire coding strand of a gene of the invention, or to only a portion thereof. Alternatively, antisense polynucleotides or ribozymes can be complementary to a noncoding region of the coding strand of a gene of the invention. The antisense polynucleotides or ribozymes can be constructed using chemical synthesis and enzymatic ligation reactions using procedures well known in the art. The nucleoside linkages of chemically synthesized polynucleotides can be modified to enhance their ability to resist nuclease-mediated degradation, as well as to increase their sequence specificity. Such linkage modifications include, but are not limited to, phosphorothioate, methylphosphonate, phosphoroamidate, boranophosphate, morpholino, and peptide nucleic acid (PNA) linkages (Galderisi et al., *supra*; Heasman (2002) *Dev. Biol.* 243:209-14; Micklefield (2001) *Curr. Med. Chem.* 8:1157-79). Alternatively, these molecules can be produced biologically using an expression vector into which a polynucleotide of the present invention has been subcloned in an antisense (i.e., reverse) orientation.

[0077] The inhibitory polynucleotides of the present invention also include triplex-forming oligonucleotides (TFOs) which bind in the major groove of duplex DNA with high specificity and affinity (Knauert and Glazer (2001) *Hum. Mol. Genet.* 10:2243-51). Expression of the genes of the present invention can be inhibited by targeting TFOs complementary to the regulatory regions of the genes (i.e., the promoter and/or enhancer sequences) to form triple helical structures that prevent transcription of the genes.

[0078] In a preferred embodiment, the inhibitory polynucleotide of the present invention is a short interfering RNA (siRNA). siRNAs are short (preferably 19-25 nucleotides; most preferably 19 or 21 nucleotides), double-stranded RNA molecules that cause sequence-specific degradation of target mRNA. This degradation is known as RNA interference (RNAi) (e.g., Bass (2001) *Nature*

411:428-29). Originally identified in lower organisms, RNAi has been effectively applied to mammalian cells and has recently been shown to prevent fulminant hepatitis in mice treated with siRNAs targeted to *Fas* mRNA (Song et al. (2003) *Nature Med.* 9:347-51).

[0079] The siRNA molecules of the present invention can be generated by annealing two complementary single-stranded RNA molecules together (one of which matches a portion of the target mRNA) (Fire et al., U.S. Patent No. 6,506,559) or through the use of a single hairpin RNA molecule which folds back on itself to produce the requisite double-stranded portion (Yu et al. (2002) *Proc. Natl. Acad. Sci. USA* 99:6047-52). The siRNA molecules can be chemically synthesized (Elbashir et al. (2001) *Nature* 411:494-98) or produced by *in vitro* transcription using single-stranded DNA templates (Yu et al., *supra*). Alternatively, the siRNA molecules can be produced biologically, either transiently (Yu et al., *supra*; Sui et al. (2002) *Proc. Natl. Acad. Sci. USA* 99:5515-20) or stably (Paddison et al. (2002) *Proc. Natl. Acad. Sci. USA* 99:1443-48), using an expression vector(s) containing the sense and antisense siRNA sequences. Recently, reduction of levels of target mRNA in primary human cells, in an efficient and sequence-specific manner, was demonstrated using adenoviral vectors that express hairpin RNAs, which are further processed into siRNAs (Arts et al. (2003) *Genome Res.* 13:2325-32).

[0080] The siRNA molecules targeted to the polynucleotides of the present invention can be designed based on criteria well known in the art (e.g., Elbashir et al. (2001) *EMBO J.* 20:6877-88). For example, the target segment of the target mRNA preferably should begin with AA (most preferred), TA, GA, or CA; the GC ratio of the siRNA molecule preferably should be 45-55%; the siRNA molecule preferably should not contain three of the same nucleotides in a row; the siRNA molecule preferably should not contain seven mixed G/Cs in a row; and the target segment preferably should be in the ORF region of the target mRNA and preferably should be at least 75 bp after the initiation ATG and at least 75 bp before the stop codon. Based on these criteria, preferred siRNA molecules of the present invention have been designed and are shown in FIG. **1**. Other siRNA

molecules targeted to the polynucleotides of the present invention can be designed by one of ordinary skill in the art using the aforementioned criteria or other known criteria (e.g., Reynolds et al. (2004) *Nature Biotechnol.* 22:326-30).

[0081] Altered expression of the genes of the present invention in a cell or organism may also be achieved through the creation of nonhuman transgenic animals into whose genomes polynucleotides of the present invention have been introduced. Such transgenic animals include animals that have multiple copies of a gene (i.e., the transgene) of the present invention. A tissue-specific regulatory sequence(s) may be operably linked to the transgene to direct expression of a polypeptide of the present invention to particular cells or a particular developmental stage. Methods for generating transgenic animals via embryo manipulation and microinjection, particularly animals such as mice, have become conventional and are well known in the art (e.g., Bockamp et al. (2002) *Physiol. Genomics* 11:115-32).

[0082] Altered expression of the genes of the present invention in an organism may also be achieved through the creation of animals whose endogenous genes corresponding to the polynucleotides of the present invention have been disrupted through insertion of extraneous polynucleotide sequences (i.e., a knockout animal). The coding region of the endogenous gene may be disrupted, thereby generating a nonfunctional protein. Alternatively, the upstream regulatory region of the endogenous gene may be disrupted, resulting in the altered expression of the still-functional protein. Methods for generating knockout animals include homologous recombination and are well known in the art (e.g., Wolfer et al. (2002) *Trends Neurosci.* 25:336-40).

[0083] The isolated polynucleotides of the present invention may be operably linked to an expression control sequence such as the pMT2 and pED expression vectors for recombinant production of the polypeptides of the present invention. General methods of expressing recombinant proteins are well known in the art.

[0084] A number of cell types may act as suitable host cells for recombinant expression of the polypeptides of the present invention. Mammalian host cells

include, e.g., COS cells, CHO cells, 293 cells, A431 cells, 3T3 cells, CV-1 cells, HeLa cells, L cells, BHK21 cells, HL-60 cells, U937 cells, HaK cells, Jurkat cells, normal diploid cells, cell strains derived from *in vitro* culture of primary tissue, and primary explants.

[0085] Alternatively, it may be possible to recombinantly produce the polypeptides of the present invention in lower eukaryotes such as yeast or in prokaryotes. Potentially suitable yeast strains include *Saccharomyces cerevisiae*, *Schizosaccharomyces pombe*, *Kluyveromyces* strains, and *Candida* strains. Potentially suitable bacterial strains include *Escherichia coli*, *Bacillus subtilis*, and *Salmonella typhimurium*. If the polypeptides of the present invention are made in yeast or bacteria, it may be necessary to modify them by, e.g., phosphorylation or glycosylation of appropriate sites, in order to obtain functionality. Such covalent attachments may be accomplished using well-known chemical or enzymatic methods.

[0086] The polypeptides of the present invention may also be recombinantly produced by operably linking the isolated polynucleotides of the present invention to suitable control sequences in one or more insect expression vectors, such as baculovirus vectors, and employing an insect cell expression system. Materials and methods for baculovirus/Sf9 expression systems are commercially available in kit form (e.g., the MaxBac® kit, Invitrogen, Carlsbad, CA).

[0087] Following recombinant expression in the appropriate host cells, the polypeptides of the present invention may then be purified from culture medium or cell extracts using known purification processes, such as gel filtration and ion exchange chromatography. Purification may also include affinity chromatography with agents known to bind the polypeptides of the present invention. These purification processes may also be used to purify the polypeptides of the present invention from natural sources.

[0088] Alternatively, the polypeptides of the present invention may also be recombinantly expressed in a form that facilitates purification. For example, the polypeptides may be expressed as fusions with proteins such as maltose-binding

protein (MBP), glutathione-*S*-transferase (GST), or thioredoxin (TRX). Kits for expression and purification of such fusion proteins are commercially available from New England BioLabs (Beverly, MA), Pharmacia (Piscataway, NJ), and Invitrogen (Carlsbad, CA), respectively. The polypeptides of the present invention can also be tagged with a small epitope and subsequently identified or purified using a specific antibody to the epitope. A preferred epitope is the FLAG epitope, which is commercially available from Eastman Kodak (New Haven, CT).

[0089] The polypeptides of the present invention may also be produced by known conventional chemical synthesis. Methods for chemically synthesizing the polypeptides of the present invention are well known to those skilled in the art. Such chemically synthetic polypeptides may possess biological properties in common with the natural, purified polypeptides, and thus may be employed as biologically active or immunological substitutes for the natural polypeptides.

[0090] The polypeptides of the present invention also encompass molecules that are structurally different from the disclosed polypeptides (e.g., which have a slightly altered sequence), but which have substantially the same biochemical properties as the disclosed polypeptides (e.g., are changed only in functionally nonessential amino acid residues). Such molecules include naturally occurring allelic variants and deliberately engineered variants containing alterations, substitutions, replacements, insertions, or deletions. Techniques and kits for such alterations, substitutions, replacements, insertions, or deletions are well known to those skilled in the art.

[0091] Antibody molecules to the polypeptides of the present invention may be produced by methods well known to those skilled in the art. For example, monoclonal antibodies can be produced by generation of hybridomas in accordance with known methods. Hybridomas formed in this manner are then screened using standard methods, such as enzyme-linked immunosorbent assay (ELISA), to identify one or more hybridomas that produce an antibody that specifically binds with the polypeptides of the present invention.

[0092] A full-length polypeptide of the present invention may be used as the immunogen, or, alternatively, antigenic peptide fragments of the polypeptides may be used. An antigenic peptide of a polypeptide of the present invention comprises at least seven continuous amino acid residues and encompasses an epitope such that an antibody raised against the peptide forms a specific immune complex with the polypeptide. Preferably, the antigenic peptide comprises at least 10 amino acid residues, more preferably at least 15 amino acid residues, even more preferably at least 20 amino acid residues, and most preferably at least 30 amino acid residues.

[0093] As an alternative to preparing monoclonal antibody-secreting hybridomas, a monoclonal antibody to a polypeptide of the present invention may be identified and isolated by screening a recombinant combinatorial immunoglobulin library (e.g., an antibody phage display library) with a polypeptide of the present invention to thereby isolate immunoglobulin library members that bind to the polypeptide. Techniques and commercially available kits for generating and screening phage display libraries are well known to those skilled in the art. Additionally, examples of methods and reagents particularly amenable for use in generating and screening antibody display libraries can be found in the literature.

[0094] Polyclonal sera and antibodies may be produced by immunizing a suitable subject with a polypeptide of the present invention. The antibody titer in the immunized subject may be monitored over time by standard techniques, such as with ELISA using immobilized marker protein. If desired, the antibody molecules directed against a polypeptide of the present invention may be isolated from the subject or culture media and further purified by well known techniques, such as protein A chromatography, to obtain an IgG fraction.

[0095] Fragments of antibodies to the polypeptides of the present invention may be produced by cleavage of the antibodies in accordance with methods well known in the art. For example, immunologically active F(ab') and $F(ab')_2$ fragments may be generated by treating the antibodies with an enzyme such as pepsin.

[0096] Additionally, chimeric, humanized, and single-chain antibodies to the polypeptides of the present invention, comprising both human and nonhuman

portions, may be produced using standard recombinant DNA techniques. Humanized antibodies may also be produced using transgenic mice that are incapable of expressing endogenous immunoglobulin heavy and light chain genes, but that can express human heavy and light chain genes.

[0097] The polynucleotides and polypeptides of the present invention may also be used in screening assays to identify pharmacological agents or lead compounds for agents capable of modulating HIPK4 activity, either substrate binding and/or kinase activity. Such screening assays are well known in the art (e.g., Turek et al. (2001) *Anal. Biochem.* 299:45-53). For example, samples containing HIPK4 (either natural or recombinant) can be contacted with one of a plurality of test compounds (either small organic molecules or biological agents), and the activity of HIPK4 in each of the treated samples can be compared to the activity of HIPK4 in untreated samples or in samples contacted with different test compounds to determine whether any of the test compounds provides: (1) a substantially decreased level of HIPK4 activity, thereby indicating an inhibitor of HIPK4 activity, or (2) a substantially increased level of HIPK4 activity, thereby indicating an activator of HIPK4 activity. In a preferred embodiment, the identification of test compounds capable of modulating HIPK4 activity is performed using high-throughput screening assays, such as provided by BIACORE® (Biacore International AB, Uppsala, Sweden), BRET (bioluminescence resonance energy transfer), and FRET (fluorescence resonance energy transfer) assays, as well as ELISA.

[0098] The present invention is illustrated by the following examples related to human, mouse, monkey and rat cDNAs, designated human HIPK4 cDNA, mouse HIPK4 cDNA, monkey HIPK4 cDNA, and rat HIPK4 cDNA, respectively, encoding novel protein kinase polypeptides designated HIPK4.

EXAMPLES

[0099] The Examples which follow are set forth to aid in the understanding of the invention but are not intended to, and should not be construed to, limit its scope in

any way. The Examples do not include detailed descriptions of conventional methods, such as those employed in the construction of vectors and plasmids, the insertion of genes encoding polypeptides into such vectors and plasmids, the introduction of such vectors and plasmids into host cells, or the expression of polypeptides from such vectors and plasmids in host cells. Such methods are well known to those of ordinary skill in the art and are described in numerous publications.

EXAMPLE 1

Identification of HIPK4 DNA Sequences

Example 1.1 Identification of Human HIPK4 Genomic and cDNA Sequences

[0100] Human HIPK4 was initially predicted by structural-based genome data mining using novel computational techniques based on bioinformatics principles described in Baxevanis and Ouellette, eds., *Bioinformatics: A Practical Guide to the Analysis of Genes and Proteins*, Wiley InterScience, New York (2001), herein incorporated by reference in its entirety. Briefly, X-ray crystal structures of the catalytic domains of known mammalian and yeast serine/threonine and tyrosine protein kinases were collected from the SCOP database (http://scop.berkeley.edu/) and aligned according to their structural identities/similarities using the ProCeryon package (http://www.proceryon.com/). This alignment was converted into a "scoring matrix" which carries the structural profile of the kinase catalytic domains. DNA sequences encoding the predicted protein sequences aligning to the scoring matrix were extracted from public human genomic sequence databases, such as Celera and GenBank. The extracted nucleic acid sequences were clustered to eliminate repetitive entries. The putative kinase domains were then sequentially run through a series of queries and filters to identify novel protein kinase sequences. Specifically, the identified sequences were used to search a nucleotide and amino acid repository of known human protein kinases using BLASTN and BLASTX. The output was parsed into a spreadsheet to facilitate elimination of known genes by manual inspection. The selected hits were then queried using

BLASTN against the NCBI *nr* and *est* databases to confirm their novelty and also to select the closest homologs.

[0101] Extensions of the partial DNA sequences were performed using the Genewise program to predict potential open reading frames based on homology to the closest homologs. Genewise requires two input sequences: the homologous protein and the genomic DNA containing the gene of interest. The homologs were identified by BLASTP searches of the NCBI *nr* protein database with the novel kinase hits described above. The genomic DNA was identified by BLASTN searches of the Celera and GenBank databases. The extended "virtual" cDNA sequences were then used to isolate the corresponding physical clones from cDNA libraries.

[0102] One such virtual cDNA sequence identified was homologous to known HIPKs of different species. This virtual cDNA sequence was used to isolate a physical clone with an open reading frame of 1848 bp (coding sequence of 1851 bp) from a human full-length testis library using GeneTrapper technology (Invitrogen, Carlsbad, CA). This novel HIPK-like clone was termed human HIPK4. The human HIPK4 cDNA sequence, and its deduced amino acid sequence, are set forth in SEQ ID NOs: 1 and 2, respectively. Based on known kinases, the 616 amino acid human HIPK4 polypeptide (FIG. **2A**) is predicted to have an ATP binding domain ranging from amino acids 17-40 (FIG. **2B**), a serine/threonine binding domain ranging from amino acids 132-144 (FIG. **2C**), and a protein kinase domain ranging from amino acids 11-347 (FIG. **2D**).

[0103] In additional experiments, isolation and sequencing of clones from a human fetal brain cDNA library corresponding to this HIPK-like sequence also revealed an open reading frame of 1848 bp (coding sequence of 1851 bp); in some clones, the sequence was identical to that isolated from the testis library, whereas in other clones a polymorphism was identified (G at position 905 changed to A, resulting in arginine at position 302 changed to glutamine). In brief detail, primer oligonucleotides were synthesized (based on virtual cDNA sequence) and used in a PCR with human fetal brain cDNA as template. The human fetal brain cDNA

library was utilized per manufacturer's instructions (Clontech, Palo Alto, CA). The sequences of the forward (SEQ ID NO:9) and reverse (SEQ ID NO:10) primers for PCR were:

5' AACCGCATCATCAAGAACGAG 3' (forward primer)

5' GTCAGGGAAGGTTAGCCGACT 3' (reverse primer).

A product of the expected size was isolated, and the DNA sequence was determined and found to be identical to the predicted sequence for that region. Additional primer oligonucleotides were synthesized and used in RACE (rapid amplification of cDNA ends) reactions in an attempt to identify the remaining portions of the cDNA. The forward (sense) primer was used in RACE reactions to identify the 3' end of the sequence; the reverse (antisense) primer was used to identify the 5' end. The sequences of the forward (SEQ ID NO:11) and reverse (SEQ ID NO:12) primers for the RACE reactions were:

5' AGACGAAGGTGCGCCCATTGGAG 3' (forward primer)

5' CTGGCGGATCCGAAGTCAATCAC 3' (reverse primer).

[0104] Comparison of the human HIPK4 cDNA sequences with the genomic DNA used for the GeneWise extension described above revealed that the human HIPK4 locus, which maps to human chromosome 19q13.1 (position numbers 38656144-38668459, according to Celera mapping), contains 4 exons and 3 introns, with 5' and 3' regions flanking the start and stop codons, respectively (see Table 3, below). The human HIPK4 genomic DNA sequence is set forth in SEQ ID NO:3.

TABLE 3

Region in SEQ ID NO:3	Sequence Attribute	Length (nt)	Position in SEQ ID NO:1
1-2000	5'-sequence[1]	2000	-
2001-2465	Exon 1	465	1-465
2466-7762	Intron 1	5297	-
7763-8119	Exon 2	357	466-822
8120-10734	Intron 2	2615	-
10735-11580	Exon 3	846	823-1668

11581-12133	Intron 3	553	-
12134-12313	Exon 4	180	1669-1848
12314-12316	Stop	3	1849-1851
12317-14316	3'-sequence[2]	2000	-

[1] 5'-sequence includes 5'-UTR (untranslated region) and/or genomic sequences

[2] 3'-sequence includes 3'-UTR (untranslated region) and/or genomic sequences

[0105] The human HIPK4 polymorphism identified in a fetal brain library (see above) was also found in a public database. A search of public single nucleotide polymorphism (SNP) databases revealed that human HIPK4 contains 25 SNPs. Only one of the SNPs in the public databases occurs in the coding region of human HIPK4 (G/A at position 10817 in SEQ ID NO:3 (in exon 3), equivalent to position 905 in SEQ ID NO:1).

[0106] CLUSTAL W sequence alignment analysis revealed that the deduced amino acid sequence of human HIPK4 shows significant homology to the kinase catalytic domains of known HIPKs (FIG. **6**). The predicted kinase catalytic domain of human HIPK4 (FIG. **2D**) has the highest homology to the kinase catalytic domains of human HIPK2 and HIPK3 (~50% identity; ~66% similarity), but it is also homologous to the kinase catalytic domains of other known HIPKs, including mouse HIPK1, HIPK2, and HIPK3, rat HIPK3, *C. elegans* HIPK1, and *S. cerevisiae* yak1. Interestingly, human HIPK4 lacks the homeodomain-interacting domain of the known HIPKs, which is a family signature of these kinases (e.g., Kim et al. (1998) *J. Biol. Chem.* 273:25875-79).

[0107] Human HIPK2 has been predicted to be a member of the dual-specificity tyrosine phosphorylated and regulated kinase (DYRK) family based on conservation of their kinase catalytic domains (Hofmann et al. (2000) *Biochimie* 82:1123-27). DYRKs are serine/threonine kinases believed to be involved in the regulation of growth and development, particularly in the brain (Himpel et al. (2000) *J. Biol. Chem.* 275:2431-38). Mutations in the *Drosophila* DYRK homolog, protein kinase MNB, results in specific defects in the development of the central nervous system (Tejedor et al. (1995) *Neuron* 14:287-301). Likewise,

overexpression of the human DYRK homolog, DYRK1A, which maps to the "Down syndrome critical region" of chromosome 21, causes neurodevelopmental delay, motor abnormalities, and cognitive deficits in mice similar to those seen in Down syndrome (Altafaj et al. (2001) *Hum. Mol. Genet.* 10:1915-23). Based on the strong homology of HIPK4 to known HIPKs at the kinase catalytic domain, HIPK4 is also predicted to be a member of the DYRK kinase family. CLUSTAL W sequence alignment analysis of the deduced amino acid sequence of human HIPK4 and known members of the DYRK family revealed significant homology at the kinase catalytic domain (FIG. **7**). In particular, human HIPK4 possesses the consensus sequence, Y - hydrophobic aliphatic residue - – - T/S - R -aromatic residue - Y - R - S/A - P - E (see FIG. **7**, amino acids 168-178), thought to confer proline specificity at the P + 1 site in DYRK substrates (see Himpel et al., *supra*).

Example 1.2 Identification of Mouse HIPK4 Genomic and cDNA Sequences

[0108] A mouse HIPK4 virtual cDNA was extracted from public murine genomic DNA databases based on the human HIPK4 cDNA sequence. This virtual cDNA sequence was used to isolate a physical clone, with an open reading frame of 1848 bp (coding sequence of 1851 bp), from a mouse full-length testis library using GeneTrapper technology (Invitrogen, Carlsbad, CA). This mouse HIPK4 cDNA sequence, which shares 87% identity with the human HIPK4 cDNA sequence, is set forth in SEQ ID NO:4. The deduced amino acid sequence of mouse HIPK4, which is 87% identical and 89% similar to the deduced amino acid sequence of human HIPK4, is set forth in SEQ ID NO:5. Like human HIPK4, the 616 amino acid mouse HIPK4 polypeptide (FIG. **3A**) is predicted to have an ATP binding domain ranging from amino acids 17-40 (FIG. **3B**), a serine/threonine binding domain ranging from amino acids 132-144 (FIG. **3C**), and a protein kinase domain ranging from amino acids 11-347 (FIG. **3D**).

[0109] Similar to human HIPK4, the deduced amino acid sequence of mouse HIPK4 shows significant homology to the kinase catalytic domains of known HIPKs (FIG. **6**). The predicted kinase catalytic domain of mouse HIPK4 (FIG. **3D**) has the highest homology to the kinase catalytic domains of mouse HIPK1,

HIPK2, and HIPK3 (50% identity; 66% similarity), but it is also homologous to the kinase catalytic domains of other know HIPKs, including human HIPK1 and HIPK2, rat HIPK3, *C. elegans* HIPK1, and *S. cerevisiae* yak1. Like human HIPK4, mouse HIPK4 lacks the homeodomain-interacting domain of the known HIPKs (FIG. **6**) and contains the proline specificity consensus sequence of known DYRKs (FIG. **7**).

[0110] Comparison of the mouse HIPK4 cDNA sequence with the genomic DNA used to extract the "virtual" cDNA described above revealed that the mouse HIPK4 locus, which maps to mouse chromosome 7A3 (position numbers 19103968-19115318, according to Celera mapping), contains 4 exons and 3 introns (see Table 4, below). A comparison of Table 3 and Table 4 shows that exonic size and intronic position are well conserved between the human and mouse HIPK4 DNA sequences. The mouse HIPK4 genomic DNA sequence is set forth in SEQ ID NO:6.

TABLE 4

Region in SEQ ID NO:6	Sequence Attribute	Length (nt)	Position in SEQ ID NO:4
1-2000	5'-sequence[1]	2000	-
2001-2465	Exon 1	465	1-465
2466-6779	Intron 1	4314	-
6780-7136	Exon 2	357	466-822
7137-7432	Intron 2	296	-
7433-8281	Exon 3	849	823-1671
8282-9171	Intron 3	890	-
9172-9348	Exon 4	177	1672-1848
9349-9351	Stop	3	1849-1851
9352-11351	3'-sequence[2]	2000	-

[1] 5'-sequence includes 5'-UTR (untranslated region) and/or genomic sequences

[2] 3'-sequence includes 3'-UTR (untranslated region) and/or genomic sequences

[0111] A search of public SNP databases revealed that mouse HIPK4 contains 12 SNPs, of which 2 (both silent) occur in the coding region of mouse HIPK4 (A/G at position 2045 in SEQ ID NO:6 (in exon 1), equivalent to position 45 in SEQ ID NO:4; and G/C at position 2444 in SEQ ID NO:6 (in exon 1), equivalent to position 444 in SEQ ID NO:4).

Example 1.3 Identification of Monkey HIPK4 cDNA Sequence

[0112] The cDNA sequence of human HIPK4 was used to search against public nucleic acid databases using BLASTN. An open reading frame of 1848 bp (coding sequence of 1851 bp) was identified that corresponded to *Macaca fascicularis* (crab-eating macaque) testis cDNA clone QtsA-20664 (GenBank AB074449). This monkey HIPK4 ortholog, which shares 96.2% identity with the human HIPK4 cDNA sequence and 86% identity with the mouse HIPK4 cDNA, is set forth in SEQ ID NO:7. The deduced amino acid sequence of monkey HIPK4, which is 97.1% identical and 97.7% similar to the deduced amino acid sequence of human HIPK4, and 87% identical and 90% similar to the deduced amino acid sequence of mouse HIPK4, is set forth in SEQ ID NO:8. Like human and mouse HIPK4, the 616 amino acid monkey HIPK4 polypeptide (FIG. **4A**) is predicted to possess an ATP binding domain ranging from amino acids 17-40 (FIG. **4B**), a serine/threonine binding domain ranging from amino acids 132-144 (FIG. **4C**), and a protein kinase domain ranging from amino acids 11-347 (FIG. **4D**).

[0113] Similar to human and mouse HIPK4, the deduced amino acid sequence of monkey HIPK4 shows significant homology to the kinase catalytic domains of known HIPKs (FIG. **6**). The predicted kinase catalytic domain of monkey HIPK4 (FIG. **4D**) has the highest homology to the kinase catalytic domains of human HIPK2 and HIPK3 (~50% identity, ~66% similarity), but it is also homologous to the kinase catalytic domains of other known HIPKs, including mouse HIPK1, HIPK2, and HIPK3, rat HIPK3, *C. elegans* HIPK1, and *S. cerevisiae* yak1. Like human and mouse HIPK4, monkey HIPK4 lacks the homeodomain-interacting domain of the known HIPKs (FIG. **6**) and contains the proline specificity consensus sequence of known DYRKs (FIG. **7**).

Example 1.4 Identification of Rat HIPK4 cDNA Sequence

[0114] A search of public nucleic acid databases using the human HIPK4 cDNA sequence also identified an open reading frame of 1848 bp (coding sequence of 1851 bp) that corresponded to a predicted *Rattus norvegicus* sequence (GenBank XM218355) similar to monkey HIPK4 identified in Example 1.3 above. Three ESTs (GenBank BF543284, BF389548, and AI716144) supported regions of the predicted sequence, including the final predicted exon and the 3' UTR, suggesting that this rat sequence is an actual expressed HIPK4. This rat HIPK4 ortholog, which shares 83% identity with the human HIPK4 cDNA sequence, 95% identity with the mouse HIPK4 cDNA, and 84% identity with the monkey HIPK4 cDNA, is set forth in SEQ ID NO:16. The deduced amino acid sequence of rat HIPK4, which is 87% identical and 88% similar to the deduced amino acid sequence of human HIPK4, 98% identical and 98% similar to the deduced amino acid sequence of mouse HIPK4, and 87% identical and 89% similar to the deduced amino acid sequence of monkey HIPK4, is set forth in SEQ ID NO:17. Like human, mouse, and monkey HIPK4, the 616 amino acid rat HIPK4 polypeptide (FIG. **5A**) is predicted to possess an ATP binding domain ranging from amino acids 17-40 (FIG. **5B**), a serine/threonine binding domain ranging from amino acids 132-144 (FIG. **5C**), and a protein kinase domain ranging from amino acids 11-347 (FIG. **5D**).

[0115] Similar to human, mouse, and monkey HIPK4, the deduced amino acid sequence of rat HIPK4 shows significant homology to the kinase catalytic domains of known HIPKs (FIG. **6**). The predicted kinase catalytic domain of rat HIPK4 (FIG. **5D**) has the highest homology to the kinase catalytic domains of human HIPK2 and HIPK3 (~50% identity, ~66% similarity), but it is also homologous to the kinase catalytic domains of other known HIPKs, including mouse HIPK1, HIPK2, and HIPK3, rat HIPK3, *C. elegans* HIPK1, and *S. cerevisiae* yak1. Like human, mouse, and monkey HIPK4, rat HIPK4 lacks the homeodomain-interacting domain of the known HIPKs (FIG. **6**) and contains the proline specificity consensus sequence of known DYRKs (FIG. **7**).

EXAMPLE 2

Tissue Expression of the Human HIPK4

Example 2.1 Northern Analysis

[0116] Tissue expression of HIPK4 was first assessed by Northern analysis using a Clontech Multiple Tissue Northern (MTN) Blot (Palo Alto, CA). MTN blots contain approximately 1μg of polyA$^+$ RNA/lane from twelve human tissues. The RNA is run on a denaturing formaldehyde 1.0% agarose gel, transferred to a nylon membrane, and fixed by UV irradiation.

[0117] Based upon the cDNA sequence of human HIPK4 (SEQ ID NO:2), PCR primers were designed to amplify a 521 nt fragment (SEQ ID NO:13) from human genomic DNA. The primers were designed to amplify coding sequence corresponding to exon 3 of human HIPK4, thereby avoiding the highly conserved kinase domain and reducing the possibility of nonspecific hybridization. The sequences of the forward (SEQ ID NO:14) and reverse (SEQ ID NO:15) primers were:

5' ACGAGACCACCCACTACTAC 3' (forward primer)

5' GAGATGCTCTCCTTCCTCCC 3' (reverse primer)

The amplified fragment was gel purified and sequence confirmed. The fragment was labeled with [α^{32}P]dCTP by random priming to produce the Northern probe.

[0118] The MTN blot was hybridized with 1-2x10^6 cpm/mL ^{32}P probe in QuickHyb® buffer (Stratagene, La Jolla, CA) along with 150μg denatured sonicated salmon sperm DNA at 68°C for 2-4 hours. The blot was washed with 2X SSC/1% SDS and 0.1X SSC/1% SDS multiple times at 65°C. Following the washes, the blot was exposed to film for multiple exposures. A very strong HIPK4 transcript approximately 5 kb in size was detected in brain tissue, while weaker transcripts of approximately 5 kb and 3 kb were detected in skeletal muscle.

Example 2.2 Tissue Array Analysis

[0119] The tissue expression of human HIPK4 was further analyzed using a Clontech Multiple Tissue Expression (MTE) array. MTE arrays are dot blots containing normalized loadings of polyA$^+$ RNA from 72 different human tissues and eight different control RNAs and DNAs.

[0120] The MTE blot was hybridized with 1-2x10^6 cpm/mL ^{32}P probe as produced in Example 2.1 in QuickHyb® buffer along with 150μg denatured sonicated salmon sperm DNA and 30μg denatured human Cot-1 DNA at 65°C for approximately 18 hours. The blot was washed with 2X SSC/1% SDS and 0.1X SSC/1% SDS multiple times at 65°C. Following the washes, the blot was exposed to film for multiple exposures. Prominent HIPK4 expression was detected in multiple subcortical, mid-brain regions known to be involved in age-related neurodegenerative diseases (e.g., Alzheimer's and Parkinson's) and in mood disorders (e.g., depression), including hippocampus, amygdala, and caudate nucleus. Weaker signals above background were also detected in testis, skeletal muscle, and lung.

Example 2.3 Cancer Array Analysis

[0121] The expression of human HIPK4 in various human cancers was assessed using a Clontech Cancer Profiling Array (CPA). CPAs are dot blots of 241 paired cDNA samples from tumor and adjacent normal tissue from individual patients.

[0122] The CPA blot was hybridized with 1-2x10^6 cpm/mL ^{32}P probe as produced in Example 2.1 in ExpressHyb® buffer (Clontech) along with 150μg denatured sonicated salmon sperm DNA and 30μg denatured human Cot-1 DNA at 65°C for approximately 18 hours. The blot was washed with 2X SSC/1% SDS and 0.1X SSC/1% SDS multiple times at 65°C. Following the washes, the blot was exposed to film for multiple exposures. Human HIPK4 appeared to be somewhat downregulated in kidney tumors. The results of these tissue expression studies indicate that human HIPK4 is a novel kinase that is strongly expressed in brain and may be improperly expressed in tumors. Thus, human HIPK4 is a potential target

for the development of human therapeutic agents, particularly those useful for treating neurological diseases and cancers.

EXAMPLE 3

Inhibition of HIPK4 Expression Using Antisense Oligonucleotides or RNAi

[0123] The ability of antisense oligonucleotides complementary to HIPK4 nucleic acid sequences, or siRNA duplexes (RNAi) matching HIPK4 nucleic acid sequences, to inhibit expression of HIPK4 can be assessed *in vitro* by treating cells in tissue culture that naturally or recombinantly express HIPK4. Decreases in HIPK4 RNA levels can be detected by Northern analysis or RNA protection assays or other methods known in the art, while decreases in HIPK4 protein levels can be detected by Western analysis (e.g., below) or immunoassay or other methods known in the art.

[0124] For Western analysis, cells are plated at an appropriate concentration in 96-well tissue culture-treated plates. For the SW480 colon carcinoma cell line, 30,000 cells/well is an appropriate cell concentration. The desired antisense oligonucleotide or siRNA duplex is transfected into the cells using an appropriate transfection reagent. Criteria for selecting and designing these oligonucleotides or duplexes are well known in the art (see, e.g., Elbashir et al. (2001) *EMBO J.* 20:6877-88). For SW480 cells, Lipofectamine 2000 (Invitrogen) is an appropriate transfection reagent. The following two master mixes/well are prepared in quantities sufficient to run samples in triplicate:
Mix 1: 30µl OptiMEM reduced serum media (Invitrogen) + 0.35µl Lipofectamine
Mix 2: 30µl OptiMEM + 500nM antisense oligonucleotide or 200nM siRNA duplex.
Mix 2 for control cells lacks antisense oligonucleotide or siRNA duplex.

[0125] Mix 1 is added to Mix 2 and incubated at room temperature for 20 min. While the incubation is proceeding, cells are washed with 100µl/well OptiMEM. Cells are refed with 100µl/well OptiMEM, returned to a humidified environment and incubated at 37°C, 5% CO_2.

[0126] The OptiMEM is removed from the cells and the Lipofectamine mixture containing the antisense oligonucleotide or siRNA duplex is added. The cells are incubated at 37°C, 5% CO_2, in a humidified environment for 4 h. The Lipofectamine mixture is removed and the cells refed with antibiotic-free media. The cells are incubated at 37°C, 5% CO_2, in a humidified environment for 5 days.

[0127] The media is removed, the cells are washed with 1X phosphate-buffered saline, and 120µl protein sample in Laemmli buffer is added to each well. The cells are scraped from the well, replicates are pooled, and the DNA is sheared by passing the samples through a 20-gauge needle.

[0128] Ten µl of each sample is loaded onto a 4-20% acrylamide Tris/glycine gel (Novex, Invitrogen, Carlsbad, CA). The gel is run for 2 h at a constant 90 V in Tris/glycine/SDS (TGS). The separated proteins are transferred to Hybond ECL nitrocellulose (Amersham Biosciences Corp, Piscataway, NJ) in 1X TGS, 20% methanol for 2 h at a constant 30 V using a Novex transfer unit.

[0129] The membranes are removed and rinsed in reverse-osmosis deionized water. The membranes are blocked in blocking buffer (1X Tris-buffered saline (TBS), 1% NP40, 5% nonfat dry milk) for 1 h at room temperature with shaking. A primary antibody that recognizes HIPK4 is diluted approximately 1:1000 in blocking buffer and added to the membrane at 4°C overnight with shaking.

[0130] The membrane is washed three times in wash buffer (1X TBS, 1% NP40) for 15 min at room temperature with shaking. The membrane is then washed once in 1X TBS for 5 min at room temperature with shaking. The horseradish peroxidase-conjugated secondary antibody appropriate for the primary antibody is diluted approximately 1:5000 in blocking buffer and added to the membrane for 2 h at room temperature with shaking.

[0131] The membrane is washed three times in wash buffer for 15 min at room temperature with shaking. The membrane is then washed once in 1X TBS for 5 min at room temperature with shaking. The blot is then developed using ECL reagents according to manufacturer's recommendations. A decrease in the amount

of HIPK4 protein in treated cells compared with control cells would indicate that the antisense oligonucleotide or siRNA duplex was effective in inhibiting expression of HIPK4.

EXAMPLE 4

Overexpression of Human HIPK4 Suppresses Programmed Cell Death (PCD)

[0132] Human HIPK4 cDNA expression plasmids were generated to investigate the effects of overexpression in standard cell death assays. The human HIPK4 coding sequence was amplified by PCR with primers containing appropriate restriction sites from a human fetal brain cDNA library (Clontech) and cloned into a pEGFP-N vector (Clontech) to provide a plasmid expressing the HIPK4 protein fused at its carboxy terminus to enhanced green fluorescent protein (EGFP). Transfections into HeLa cells and the human neuroblastoma line SH-SY5Y were performed by standard lipid-based protocols.

[0133] Cells expressing EGFP were scored for nuclear morphology at 48 h posttransfection to assess the effects of human HIPK4 on programmed cell death (PCD). The overexpression of human HIPK4 did not induce PCD in either SH-SY5Y cells or HeLa cells as compared to EGFP alone. Cells were also treated 48 h posttransfection with staurosporine (STS), a potent inducer of PCD, for 4 h to evaluate potential protective effects of human HIPK4 expression. Human HIPK4 inhibited the induction of PCD by STS in both SH-SY5Y cells (250nM STS) and HeLa cells (1μM STS) by 30-40%. Human HIPK4 expression also reduced PCD in HeLa cells by ~40% following 24 h treatment with 100μM etoposide, an agent with a different mechanism of action than STS. These data demonstrate that human HIPK4 has a general protective activity against apoptosis, evident in different cell lines and with different initiating injuries.

[0134] Because it was important to determine whether the protective effect of human HIPK4 was a result of protein kinase activity, amino acids lysine-40 and aspartate-136 of human HIPK4, predicted to be functionally essential for kinase activity based on homology to known kinases (Bairoch and Claverie (1988) *Nature*

331:22), were substituted by site-directed mutagenesis (serine for lysine-40, and tryptophan for aspartate-136) using the QuickChange™ System (Stratagene) according to manufacturer's recommendations. Substitution of lysine-40 and aspartate-136, individually or together, almost completely abrogated the protective effect of human HIPK4 in HeLa cells against injury by 1μM STS, indicating that the anti-PCD activity of human HIPK4 is mediated by its predicted protein kinase function.

[0135] The protective effects of human HIPK4 were also examined in a model system of neurodegeneration. Rat cerebral granular neurons (CGNs) grown in culture were transfected with an expression vector for human HIPK4/EGFP fusion protein or EGFP vector alone by a calcium phosphate method (Xia et al. (1996) *J. Neurosci.* 16:5425-36). Bcl-x_L expressed as an EGFP fusion protein served as a neuroprotective positive control (Boise et al. (1993) *Cell* 74:597-608). Transfected CGNs were injured 48 h posttransfection by transfer to growth media containing low levels of K^+ (5mM) and no serum for 24 h. CGNs expressing EGFP were scored for apoptosis by nuclear morphology. CGNs expressing HIPK4 were substantially protected from apoptotic injury as compared to vector alone (~60% greater cell survival); in fact, this level of protection was equivalent to that seen in CGNs transfected with the antiapoptotic Bcl-x_L protein.